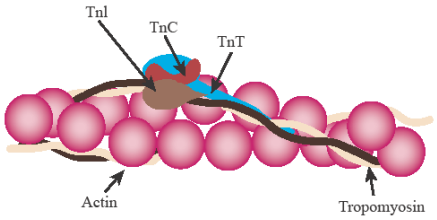
Release and Clearance Mechanisms of Cardiac Troponin



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Cover illustration: The Liver and Kidneys mediate clearance of cardiac troponin in the rat. Scientific Reports, Springer Nature 2020.

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Release and Clearance Mechanisms of Cardiac Troponin

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ABSTRACT

Myocardial infarction (MI) is often suspected when a patient presents with chest pain. MI is defined as cardiac necrosis due to ischemia, most often mediated through impaired coronary perfusion. Cardiac necrosis results in the release of myoglobin, creatine kinase and cardiac troponin (cTn) to the circulation. According to current guidelines, the MI diagnosis is, to a large extent, based on the patient's levels of cTn. This thesis examines the mechanisms of cTn release and subsequent clearance from the circulation. The trimeric cardiac troponins, troponin T (cTnT), troponin I (cTnI) troponin C (cTnC), bind to each other and via cTnT to insoluble filaments in the cardiomyocyte. Contrary to the prevailing opinion we found that a large fraction of cTnT could be released in 37°C plasma from necrotic human cardiac tissue without degradation of insoluble filaments. In contrast to myoglobin, which lacks affinity for cardiac tissue, the release of cTnT was

highly plasma volume-dependent, which could explain the delayed clearance of cTnT observed in patients with MI. We then examined the clearance of cTnT from the circulation by injecting cardiac extracts containing both myoglobin and cTnT in rats. We also examined the renal extraction of circulating cTnT by comparing the cTnT concentration in blood samples from the renal vein and an artery in heart failure patients. We found high renal extraction of cTnT and that correction for renal clearance makes the cTnT analysis slightly better at finding patients with an MI in the emergency ward. We next examined the difference in release and clearance of cTnT and cTnI using the currently most frequently used clinical assays. We found that most cTnT and cTnI released from human cardiac tissue were degradation products produced by tissue-resident proteases. We also found that cTnI was degraded and released much faster than cTnT, whereas their subsequent clearance, once they reached the circulation, did not differ between cTnT and cTnI in either rats or humans. Our data potentially explain why cTnI reaches higher levels and disappears faster than cTnT in patients with MI.

Keywords: Cardiac Troponin T, Cardiac Troponin I, biomarkers, human, myocardium, animals, kidney-dependent clearance, myocardial infarction

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SAMMANFATTNING PÅ SVENSKA

Hjärtinfarkt (MI) misstänks ofta när en patient upplever bröstsmärta. MI definieras som hjärtcellsdöd till följd av syrebrist, vilket oftast orsakas av försämrad genomblödning genom koronarkärlen. Hjärtnekros resulterar i frisättning av myoglobin, kreatinkinas och hjärttroponin till blodcirkulationen. Enligt gällande riktlinjer baseras MI-diagnosen till stor del på att mäta blodets innehåll av hjärttroponin. Denna avhandling undersöker mekanismerna för troponinernas frisättning samt nedbrytning och efterföljande elimination från cirkulationen. De trimeriska hjärttroponinerna, troponin T (cTnT), troponin I (cTnI) och troponin C (cTnC), binder till varandra och via cTnT till olösliga filament i hjärtmuskelcellen. Till skillnad från den rådande uppfattningen fann vi att en stor del av cTnT kunde frisättas i 37° plasma från nekrotisk human hjärtvävnad utan nedbrytning av olösliga filament. I motsats till myoglobin, som saknar starka bindningspartners i hjärtcellen och därför frisätts till blodet direkt, så var frisättningen av cTnT i våra experiment mycket plasmavolymberoende, vilket skulle kunna förklara den försenade frisättningen av cTnT som man ser hos patienter med MI. Därefter undersökte vi rensning av cTnT från cirkulationen genom att injicera hjärtextrakt innehållande både myoglobin och cTnT i råttor och rensningen följdes med upprepade blodprov. Vi undersökte också extraktion av cTnT via njurarna i blodcirkulationen genom att jämföra cTnT-nivåerna i blodprover från njurvenen och njurartären hos patienter med hjärtsvikt. Vi noterade omfattande cTnT clearance via njurarna och såg att korrigering för njurens extraktionsförmåga gjorde cTnT-analysen lite bättre på att hitta patienter med MI i akutvården. Därefter undersökte vi skillnaden i frisättning och clearance av cTnT och cTnI mätt med de två oftast använda kliniska mätmetoderna. Vi fann att större delen av den cTnT och cTnI som frisattes från human hjärtvävnad var nedbrytningsprodukter som produceras av proteaser i

vävnaderna. Vi fann också att cTnI degraderades och frisattes mycket snabbare än cTnT medan påföljande clearance från cirkulationen inte skilde sig åt mellan cTnT och cTnI hos varken råttor eller människor. Våra data kan potentiellt förklara varför cTnI når högre nivåer och försvinner snabbare än cTnT hos patienter med MI.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Starnberg K, Jeppsson A, Lindahl B, Hammarsten O. Revision of the Troponin T Release Mechanism from Damaged Human Myocardium. Clinical Chemistry, 2014;60,8: 1098-1104.

II. Fridén V, Starnberg K, Muslimovic A, Ricksten SE, Bjurman C, Forsgard N, Wickman A, Hammarsten O. Clearance of cardiac troponin T with and without kidney function. Clinical biochemistry, 2017; 50, 9: 468-474.

III. Starnberg K, Fridén V, Muslimovic A, Ricksten SE, Nystrom S, Forsgard N, Lindahl B, Vukusic K, Sandstedt J, Dellgren G, Hammarsten O. A Possible Mechanism behind Faster Clearance and Higher Peak Concentrations of Cardiac Troponin I Compared with Troponin T in Acute Myocardial Infarction. Clinical Chemistry, 2020; 66, 2: 333-341.

CONTENT

ABBREVIATIONS

ACC	American College of Cardiology
ACS	Acute coronary syndromes
AMI	Acute myocardial infarction
AR	Argon
CAD	Coronary artery disease
CK	Creatine kinase
CKD	Chronic kidney disease
CKMB	Creatine kinase muscle brain fraction
CRS	Cardiorenal syndrome
cTn	Cardiac troponin
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
CV	Coefficient of variation
CVD	Cardiovascular disease
ECG	Electrocardiography

ED	Emergency department
EDTA	Ethylene diamine tetra acetic acid
ER	Emergency room
ESC	European Society of Cardiology
ESRD	End stage renal disease
GFR	Glomerular filtration rate
HF	Heart failure
hs	High sensitive
ICP-MS	Inductively coupled plasma - mass spectrometry
K_D	Dissociation constant
KF	Kidney failure
MI	Myocardial infarction
Myo	Myoglobin
NSTEMI	Non-ST-elevated myocardial infarction
PAH	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
PKA	Protein kinase A
PKC	Protein kinase C

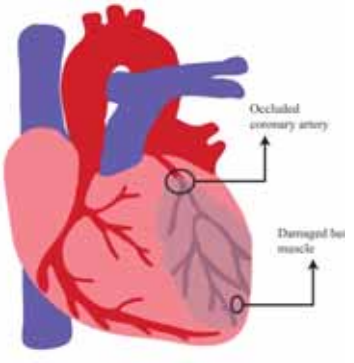
RI	Renal impairment
RPF	Renal plasma flow
RBF	Renal blood flow
SEC	Size exclusion chromatography
SHAM	Control animals
SR	Sarcoplasmic reticulum
ss	Slow skeletal
STEMI	ST-elevated myocardial infarction
URL	Upper limit detection

1 INTRODUCTION

1.1 History

According to the World Health Organization (WHO), cardiovascular diseases (CVDs) kill 17.9 million people every year, which equals 31% of all global deaths. One of the cardiovascular diseases is myocardial infarction (MI).

Figure 1. Heart with blocked blood supply and damaged heart muscle cells.



Many years have passed since the first post-mortem examinations in the late 19th century indicated a relationship between thrombotic occlusion of a coronary artery and myocardial infarction. Some years later, at the beginning of the 20th century, this relationship was observed clinically and described in greater detail, but it was still not generally accepted, due to autopsy findings of non-thrombotic arteries in 31% of MI cases [1]. In the 1950s-70s, WHO established ECG-based definitions of MI, and in the 21st century, expert committees recognized the pivotal importance of the biomarkers cardiac troponin (cTn). These committees also recognized the value of being able to measure the low levels of cTn found in healthy people and advocated the use of "healthy levels" of cTn as the cutoff for defining patients with or without MI. At that time, no commercial cTn assay was able to measure the low cTn levels found among healthy individuals with sufficient precision, which, in turn, initiated a

race among assay manufacturers to develop these assays. Now, over a decade later, several cTn assays with sufficiently good performance have been validated for clinical use. The availability of these high-sensitive (hs) cTn assays have, in turn, fostered the development of safe and rapid rule-out algorithms that have reduced the costs associated with MI care by half in some instances [2]—true medical progress.

At present, the European Society of Cardiology (ESC) and the American College of Cardiology (ACC) recommend the use of the 99th percentile of cardiac troponin T (cTnT) and cardiac troponin I (cTnI) values, defined by the cTn level that 99% of the healthy population is found below. The measurement of this value should have a precision with a coefficient of variation (CV) < 10%. Currently, the ESC "Fourth universal definition of Myocardial Infarction", published in 2018, constitute the most widely accepted guidelines in clinical use [1]. The introduction of hs- cTn assays in clinical practice has reduced the number of hospitalizations and has cut health care costs by half without affecting mortality or missed myocardial infarctions [2].

The term MI is used to describe an acute myocardial ischemic event mediated through impaired coronary perfusion. Clinical evidence of acute myocardial ischemia includes symptom presentation; *i.e.,* sudden-onset severe chest pain and signs of cardiac ischemia, specific electrocardiography (ECG) abnormalities and laboratory findings.

1.2 Myocardial damage and cardiac-specific troponins

In the myocardium itself, reduced cellular glycogen, sarcolemma disruption and relaxed myofibrils are believed to be the first ultrastructural changes related to ischemia, and are assumed to be progressive [1].

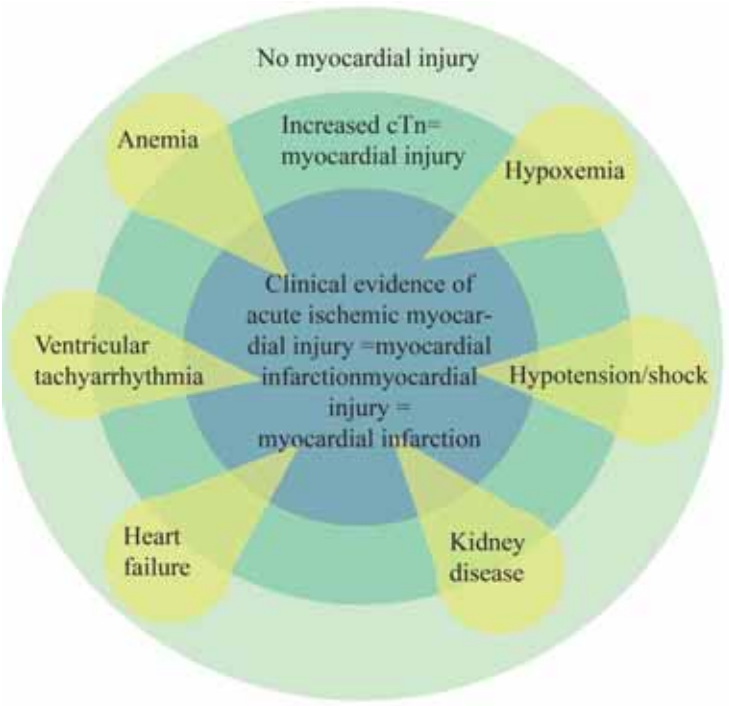


Figure 2. Spectra of myocardial injury.

It is in this state of anoxia that the myocardium releases various intracellular components; for example, cTnT and cTnI that are used as serum biomarkers for acute MI (AMI). cTn are circulating diagnostic biomarkers released by

myocardial cells during an ischemic event. If a patient has a cTnT elevation above the 99th percentile, myocardial damage should be suspected. A rise and/or fall pattern in cTnT levels over time of at least 20% in relation to the baseline value indicates that the injury should be considered acute [1]. If the hs-cTnT or hs-cTnI levels remain below the cTn assay-specific 99th percentile during the hospital stay, it is normally considered that the patient did not experience an MI.

Type 1 MI is the most common infarction type, where a rupture in an atherothrombotic coronary artery plaque causes occlusion of one or more coronary vessels [1].

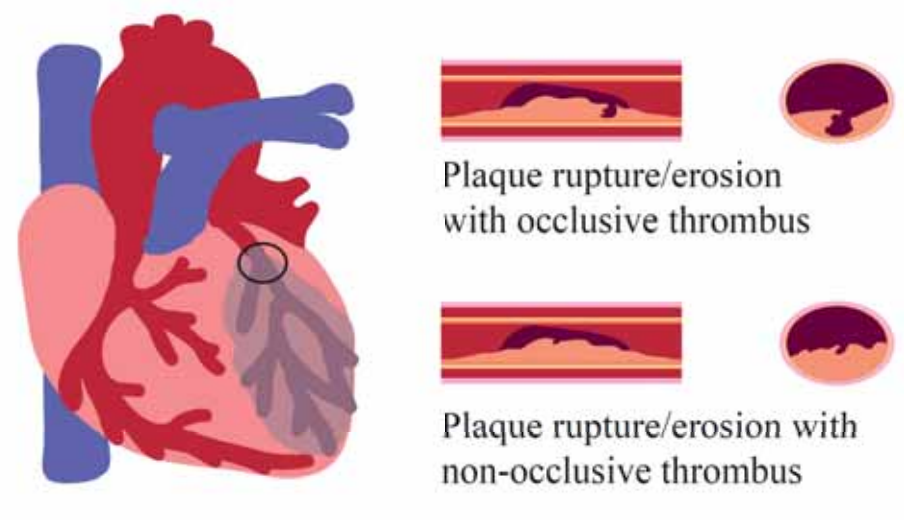


Figure 3. ***Myocardial infarction type 1.*** Occlusive or non-occlusive plaque rupture/erosion.

Hallmark signs include rapid onset, usually severe symptoms such as chest pain or dyspnea, and typically, but not limited to, ST elevation, new pathological Q waves and left bundle branch block patterns on a 12-lead ECG. Imaging evidence, usually in the form of angiography, confirms the

diagnosis, and percutaneous coronary intervention, or PCI, is the present golden standard treatment in these cases. Because of the ubiquitous pattern of ST elevation, type 1 MI is commonly referred to as ST elevation myocardial infarction (STEMI), in clinical practice. In these cases, the ECG findings are pathognomonic for transmural ischemia and the patient is usually referred directly to the angio lab for angiography and possibly intervention by percutaneous coronary intervention (PCI). In these cases, it is widely accepted that cTn testing is not needed for a diagnosis.

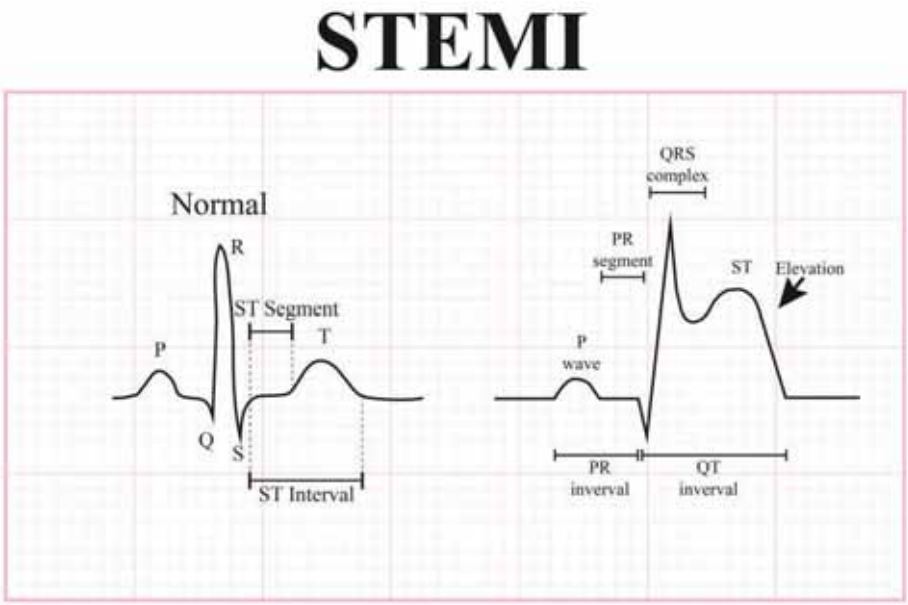


Figure 4. ***ECG curve. To the left- normal S-T interval.*** *Detection of biomarkers are needed to exclude MI in patients. To the right- elevated S-T interval due to occluded arteries*

However, coronary occlusions do not always result in ST elevation on ECG. This is because some occlusions do not leave the entire ventricle wall ischemic. In these events, the ECG often shows signs of impaired perfusion by ST segment depression or T wave inversion, but up to 50% of patients

with type 1 myocardial infarction have normal or inconclusive ESC findings. These patients are referred to as non-ST-elevation myocardial infarction (NSTEMI) patients.

The diagnosis of NSTEMI relies more heavily on cTn, both in absolute levels and with regard to how cTn levels change over time; increasing or decreasing over consecutive blood samples. Angiography is sometimes used to confirm the diagnosis, but treatment, especially in the short term, is mainly pharmacological.

In the absence of ST elevations or other pathognomonic signs of a transmural MI, the MI diagnosis relies on symptoms and elevation of cTn levels and/or findings of significant occlusion on PCI or decreased myocardial wall movement on cardiac ultrasound (UCG). These patients are often premedicated with drugs that prevent blood clotting when admitted and subjected to a sub-acute coronary angiography.

In some cases, the coronary occlusion is transient, and the ischemic periods are so short that no cTn is released. In the typical scenario, the thrombotic event is successively cleared by fibrinolysis and reformed by recoagulation. The patient often experiences sudden periods of chest pain followed by its resolution, sometimes for days before seeking medical care. This condition is called unstable angina and is often a real challenge for the clinician, as no reliable biomarker exists that can exclude this condition of transient occlusions. Fortunately, since the introduction of high-sensitive cTn assays, unstable angina is a rare but feared condition.

Type 2 MI, as opposed to Type 1, is a myocardial ischemic event, not mediated by a ruptured plaque but by progressive stenosis of the arterial lumen, which eventually reaches the pivotal supply/demand point where the

myocardium can no longer be adequately oxygenated. As such, it is on the same continuum as unstable angina. The clinical presentation is usually somewhat less dramatic, and with less ostentatious ECG abnormalities. Type 2 MI and myocardial injury are both associated with a poor outcome [1].

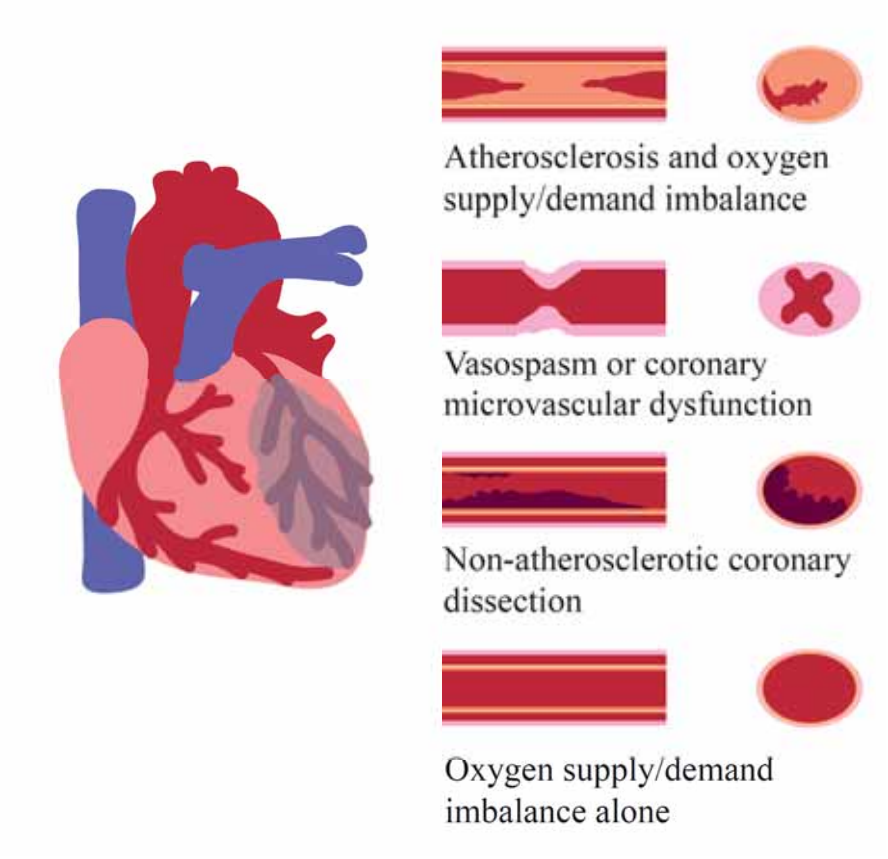


Figure 5. ***Myocardial infarction type 2.*** *Ischemic myocardial event not due to plaque rupture.*

In summary, cTn are circulating diagnostic biomarkers released by myocardial cells during an ischemic event. If a patient has a cTnT elevation above the 99th percentile, myocardial ischemia should be suspected. A rise and/or fall pattern in cTnT levels over time, of at least 20% in relation to the baseline value, indicates that the injury should be considered acute [1]. If the hs-cTnT or hs-cTnI value remains below the assay-specific 99th percentile during the hospital stay, the patient did not normally suffer a myocardial infarction (MI).

This thesis will focus on the cTn biomarkers used to include/exclude myocardial injury.

1.2.1 Gender differences in symptoms and prognosis

The incidence of MI is the same in women and men, but, on average, women are affected ten years later in life, possibly due to the protective effects of estrogen [3] that decrease the degree of atherosclerosis, left ventricular (LV) hypertrophy and cardiomyocyte apoptosis, which all tend to be less severe in women, probably resulting in the less pronounced effects on cardiomyocytes and the lower cTn release in women compared with men [4].

Following an MI, women have a lower one-year survival rate, possibly due to the underdiagnosis of women [5], but men have an overall higher risk of death and heart disease than women. This is possibly related to the twice as high levels of stable cTn levels in men compared with women [4]. This could not only be due to men having larger hearts than women. The exact underlying mechanisms behind men having higher stable cTn levels than women are not known, but estrogen is probably one factor. For some reason, the higher the

stable cTn levels, the greater the risk of future cardiac events like MI and death, which likely reflects sex-related differences in the pathobiology of CAD, with women more often having microvascular and endothelial dysfunction and/or diffuse coronary atherosclerosis [4]. This possibly adds to the "the stable troponin problem", called "myocardial injury" by the task force for the definition of myocardial infarction [1]. For instance, at the same cTn level, men and women have a similar risk, indicating that "the stable troponin problem" is a driver behind the higher risk of cardiac death in men.

Chest pain is the most common marker for both women and men, but women's coronary arteries are more delicate and often have distributed coronary atherosclerosis, while men's plaques are more of a focal nature. Women also tend to have more diffuse symptoms [6]. Silent infarctions and Takutsobu (broken heart syndrome) are more common in women [1].

1.2.2 Cardiomyocytes

About 30% of the human heart consists of cardiomyocytes, the motile cells that create the pumping function of the heart [7]. They may be branching and have one or two nuclei in the core of the cell, and the cells are thought of as a cell community, in contrast to the skeletal myocytes that are thought of as one large unit. That is, all cardiac cells are electrically interconnected. Therefore, an action potential is set off in one of these interconnected cardiac cells and a wave of contracting cardiomyocytes will spread throughout the heart. This is in strong contrast to the voluntary contraction of skeletal muscles, which are divided into functional units that contract in unison if activated by an incoming nerve signal. A visual representation of the interconnectivity of heart cells is the beating of a cardiac cell monolayer if

the cells are grown densely enough to allow a critical mass of interconnected cardiomyocytes. The contraction that can be seen in the monolayer; for example, in isolated neonatal rat cardiomyocytes, beats twice every second in the beginning and after attachment of the cells to the cell culture well, and when plated in spheres they start beating simultaneously every fifth second and can be speeded up by adding adrenalin to the cell medium where the beat frequency can be increased fivefold.

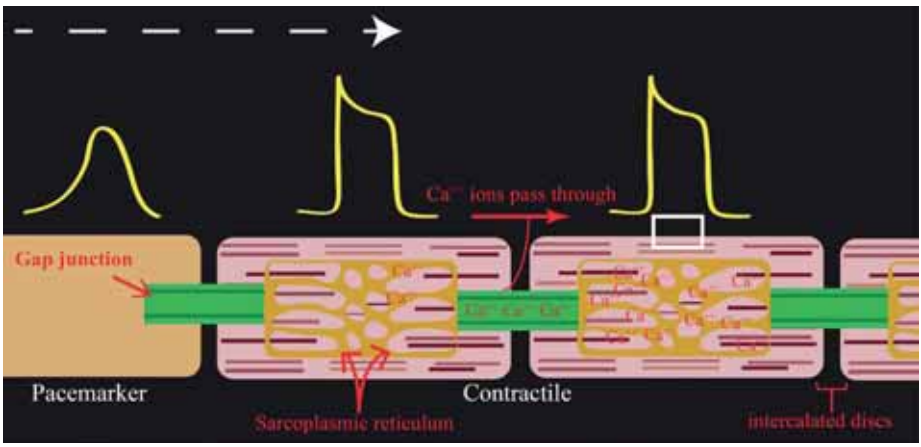


Figure 6. ***Depolarization wave.*** *Pacemaker cells initiate the electrical impulse. Depolarization wave are spreading by Ca^{2+} and other ions. Ca^{2+} containing SR releases Ca^{2+}, gap-junctions enable the ions to pass through neighboring cells, creating the heart contractions.*

The cardiomyocyte has intercalated discs and doughnut-shaped gap junctions between the cells, which allow them to communicate with each other in different ways; for instance, by passing on the depolarization wave by letting ions such as Na^{+} and Ca^{2+} pass easily. The Ca^{2+} ions from the earlier cell and from the extracellular space enter the T tubule of the new cells and bind to receptors on the sarcoplasmic reticulum (SR), which is an organelle structured like a "river delta" that stores large amounts of Ca^{2+}. When the extracellular Ca^{2+} binds to receptors on the SR, it releases large amounts of

Ca^{2+} into the cell where the myofibrils are. The myofibrils consist of sarcomeres, which are the contractile units in the cells.

The sarcomere includes thick filaments (myosin) and thin filaments (actin). Two actin filaments are wired along each other and are attached to the Z discs by desmosomes. Tropomyosin is wired around the actin filament, and a cTn complex is attached to the tropomyosin on every 7th actin molecule [8]. The cTn complex consists of three subunits: Cardiac Troponin T (cTnT), the tropomyosin-binding subunit; cardiac Troponin C (cTnC), the calcium-binding subunit, and [9] cardiac Troponin I (cTnI), the inhibiting subunit. Together they form a part of the regulated contraction within the cardiomyocyte.

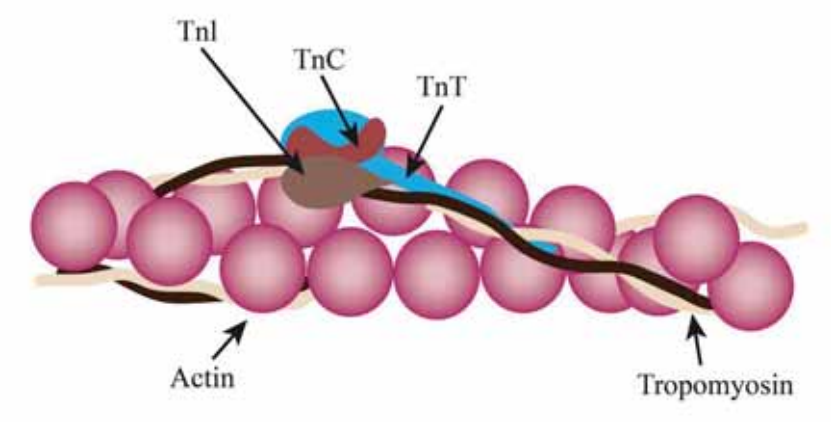


Figure 7. ***Thin filaments.*** *Tropomyosin is wired around the actin filament and on every 7th actin molecule; a troponin complex is attached to the tropomyosin.*

The cTn complex interacts with tropomyosin on the thin actin filaments. At the onset of systole Ca^{2+} binds C-terminally to cTnC which leads to a cTnC conformation switch, changing cTnI to enable and catalyze protein-protein

associations that activate the thin filaments [10] and enable cross-bridge formation between actin and myosin. The myosin starts to climb along the actin, which compresses the myocyte when millions of similar events occur. The heart starts to contract.

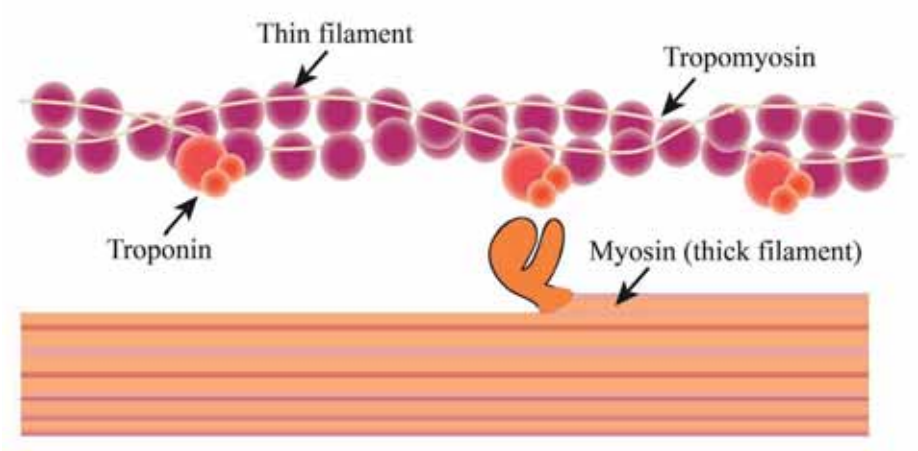


Figure 8. ***Heart contraction.*** *The troponin complex modifies tropomyosin and thereby enabling cross bridge formation between action and myosin leading to heart contraction.*

1.2.3 Cardiac Troponin and detection of cardiac damage

The cTn complex is composed of three proteins in a 1:1:1 complex of around 80kDa: cTnT (37kDa), cTnI (24kDa) and cTnC (18kDa). cTnT and cTnI are diagnostic biomarkers inblood samples when myocardial infarction is suspected. There is no clinical testing for cTnC since it is not heart-specific [11].

As far as we know, cTnI is only expressed in cardiomyocytes [12]. Something that reacts with the clinical cTnT assay is also expressed at low levels in

skeletal muscles. Despite this, the clinical performance of cTnT and cTnI analyses are very similar and are considered reliable biomarkers for cardiac damage, unlike the other older biomarkers, such as myoglobin [1] and creatine kinase muscle/brain fraction (CKMB), which are expressed at high levels in other muscle types.

When cardiac damage occurs, cTnT is released and can often be measured in the circulation for over a week. This is due, in part, to the tight binding of cTnT to the insoluble thin filaments in the necrotic cardiomyocyte. Myo and CKMB, on the other hand, lack affinity for the dead cardiomyocyte and are released faster and, hence, also disappear within a few days from the circulation, although all these biomarkers have similar half-lives of 1-2 hours [13-18] once they reach the circulation [19-21].

Since 2000, the ESC guidelines [1,22] recommend the use of cTn and high-sensitive assays as the golden standard for biomarkers in cardiovascular events, in order to rule in/rule out MI or other myocardial damage. The ESC and the Task force for the definition of myocardial infarction also recommend using the assay-dependent 99th percentile among healthy individuals for rule-out; that is, according to the latest guidelines, the patient must normally, at some time during the hospital stay, have a cTn value above the 99th percentile to get an MI diagnosis. The 99th percentile is assay-dependent, and the local laboratories were originally encouraged to find "their" 99th percentile.

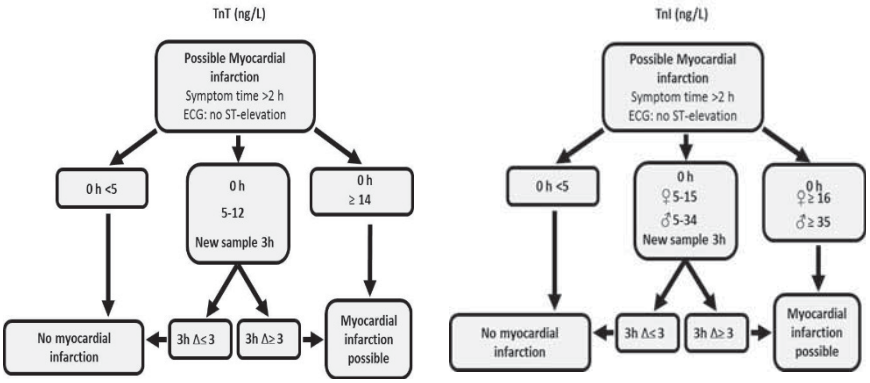


Figure 9. Triage for Roche hs-cTnT and Abbot hs-cTnI

However, several studies have now determined the 99th percentile for most of the high-sensitive cTnT and cTnI assays in clinical use [5]. The ESC guidelines recommend that the analytical limit of detection (LOD) should include the 99th percentile with a coefficient of variation (CV) < 10%, meaning that the standard deviation of repeated measurements should be less than 10% of the mean value from the repeated measurements [1].

$$CV = \frac{SD}{m} \times 100$$

Figure 10. ***Coefficient of variation.*** *SD = standard deviation, m = mean x100 to receive it in percent.*

1.2.4 Clinical Troponin Assays

The clinical assays for cTnT and cTnI that were used in this study are all designed to detect degradation products since this enhances their ability to measure low levels of cTn. Like most sandwich assays, the hs-cTnT and hs-cTnI assays use two antibodies that must colocalize on the same cTn molecule to give a signal.

The Roche hs-cTnT assay works according to the sandwich principle, in which a detector (M7) and catcher (M11.7) are used. The capture and trace antibody pair bind two epitopes located in the middle of the cTnT (amino acids 125–131 and 137-148), resulting in the detection of full-length cTnT and various degradation products [23,24].

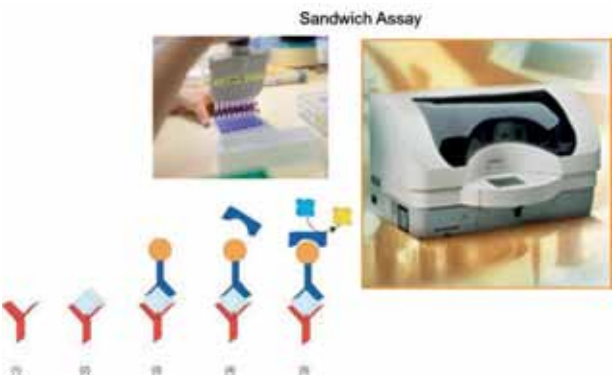


Figure 11. ***Sandwich Assay.*** *Both capture antibody and detector antibody are required to bind to enable signal.*

There are about 20 different analyses for cTnI that detect different epitopes [25]. The hs-cTnI assay from Abbot that was used in our studies uses a capture antibody that binds amino acids 24-40, and a tracer antibody that bind amino acids 41-49. This assay is also able to detect both full-length cTnI and degradation products [26].

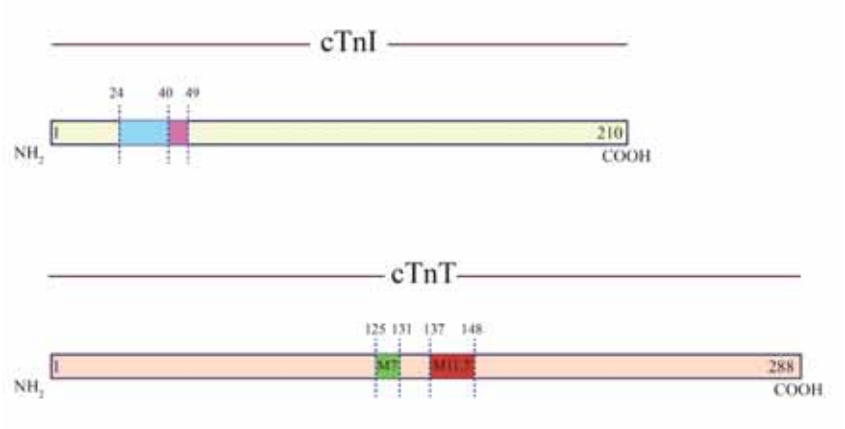


Figure 12. Epitopes for the Antibodies used in Abbot hs-cTnI and Roche hs-cTnT.

1.2.5 The diagnostic cutoff for cardiac troponin

The 99th percentile is equivalent to the maximum levels of cTn, below which 99% of the healthy population are found. The ESC recommends a CV less than 10%. Assays with a CV > 20% should not be used. To be classified as a high-sensitivity assay, the CV at the 99th percentile should be < 10 %. The hs analysis should also be able to detect cTnT levels in > 50% of the healthy population [1,27].

The patient's minimum time-dependent cTn dynamic to be included for an MI is 20%, even though the dynamics for a healthy population may vary by up to 48%. Interestingly, there is evidence that the dynamics of patients with stable increases in cTnI is 14% and 7% for cTnT [28].

The diagnostic cutoff values for cTn have been intensely debated. For instance, the "healthy population" is not representative of patients in the emergency department (ED), the population most often analyzed with cTn tests. In addition, many other conditions; for example, high age, heart failure (HF) and kidney failure (KF), all tend to cause elevated cTn levels also in the absence of an MI or other causes of cardiac necrosis. Additionally, there is no

consensus on how to define a healthy individual [29]; for example, age and comorbidities affect the 99th percentile, increasing the risk of sample-to-sample variation[30].

Most studies have used 14 ng/L as the 99th percentile cutoff value for the hs-cTnT assay, with a CV ≤ 10% at 13.5 ng/L. However, the level of hs-cTnT in patients > 60 years of age, is reported to be higher than in the population < 60 years of age, indicating that age-specific cutoffs are needed [31,32]. Sex-specific differences were published in 2009 [33]. In one study on a smaller Chinese population, it was argued that 11 ng/L of cTnT would be more beneficial to middle-age patients (40-60 years) [34]. Others publications state that their testing is similar to the manufacturer's limit [35]. Giannitsis et al. [36] studied 616 individuals and demonstrated a 10% CV of 13.0 ng/L with the 99th percentile for the entire population of 13.5 ng/L. This study also observed a significant difference (P = 0.01) between the 99th percentile of men (14.5 ng/L) and women (10.0 ng/L). When Mingels et al. used a pre-commercial version of the hs-TnT assay in 546 apparently healthy human samples, the 99th percentile URL was 16 ng/L, with significant differences between age and gender.

Furthermore, the introduction of high-sensitivity cTn assays revealed female/male differences, implying that the 99th percentile is too high for women, leading to women being underdiagnosed [37]. Furthermore, the 99th percentile reported for cTnT and cTnI assays have not been derived from the same reference population and may therefore not be biologically equivalent [5]. The ESC Task force recommend but does not require sex-specific cutoffs [1].

cTn also have a dark side. Nearly one fifth of patients arriving at the ED with cardiac symptoms have elevated levels of cTn, without manifest Acute Coronary Syndrome (ACS). The mechanism behind these cTn increases is

often unknown and may be due to various causes, including ventricular strain, myocyte trauma, impaired renal clearance and possibly release from living but stressed cardiomyocytes [12,38,39]. With slightly increased levels, the patient will often be admitted, even though there may not be an MI, leading to increasing hospital costs without obvious beneficial treatment. Whatever the underlying reason, patients presenting with a cTn elevation without a manifest ACS or other acute condition according to their medical records have a tenfold higher risk of death and heart disease [40], a prognosis even worse than for patients presenting with myocardial infarction. Since the underlying mechanism of this "stable troponin problem" is not fully understood, we still lack evidence-based treatment for these poor prognosis patients [12].

1.3 Release of cardiac troponin from necrotic cardiomyocytes

It is presumed that most cTnT is irreversibly bound to the myofibril and only released following the degradation of myofibrils in necrotic cardiomyocytes, resulting in sustained increases in circulating cTnT. These assumptions are based on the inability to extract more than 5-10% of the total amount of cTnT from human cardiac tissue in cold low-salt extraction buffers, which indeed are able to extract all myoglobin and creatine kinase from the same tissue [41]. The cold low-salt buffers were designed as wash buffers as a first step in the cTnT purification protocol originally described by Potter et al. [42]. As cTnT binds tightly to insoluble thin filaments in the cardiac tissue, only 5-10% of the total cTnT content of the cardiac tissue was found in this buffer [42]. This

inability to extract cTnT was taken as evidence that 5% of cTnT was "cytosolic" and that only this amount of cTnT could be released without degradation of myofibrils. According to this model, the slow washout of cTnT after an MI was due to the degradation of insoluble myofibrils as granulocytes accumulated in the necrotic tissue. The model involving 5% of cTnT being free in the cytosol and 95% of cTnT being released upon tissue degradation has prevailed in textbooks since then [41].

1.3.1 Simple diffusion, Dissociation Constant, K_D

If a protein is structurally bound; for instance, as a part of a polymer like actin, it will stay attached to its binding partner regardless of the surrounding concentration and only detach when degradation or depolymerization occurs. Non-structural proteins can be attached to structural proteins via their affinity for binding partners in an equilibrium with simple diffusion, such as cTnT bound to tropomyosin/actin filaments [43]. The dissociation constant ($\mathbf{K_D}$) is the concentration of the ligand at equilibrium, when half of the ligand's binding sites are occupied by ligands.

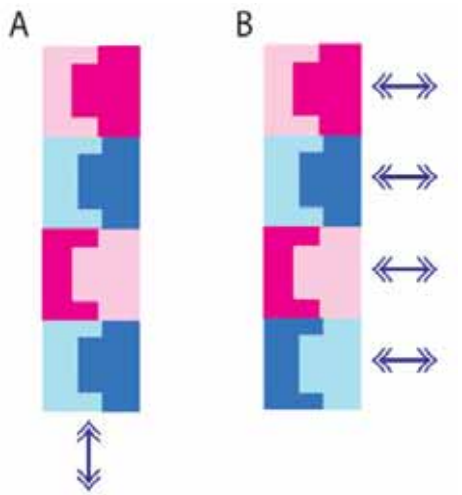


Figure 13. ***Structurally and non-structurally bound proteins.*** *(A)- Structurally bound proteins such as Actin polymers, will stay attached to its binding partner regardless of the surrounding concentration and only detach when degradation or depolymerization occurs (B)- Non-structural proteins can be attached to structural proteins via their affinity for binding partners in an equilibrium with simple diffusion, such as cTnT bound to tropomyosin/actin filaments.*

The smaller the $\mathbf{K_D}$, the higher the affinity for the binding. It follows that if the concentration of the ligand, like cTnT, is a 1000-fold higher than the K_D, almost all binding sites on the tropomyosin/actin filaments will be occupied. On the other hand, if the cTnT concentration is a 1000-fold lower than the K_D, most cTnT will not be bound to available binding sites on tropomyosin/actin filaments. According to the concentration-dependent behavior of protein-protein interactions, it will be possible, in principle, to extract all cTnT from its binding sites in the tropomyosin/actin filaments by increasing the extraction volume, thereby decreasing the cTnT concentration.

1.3.2 Troponin degradation products

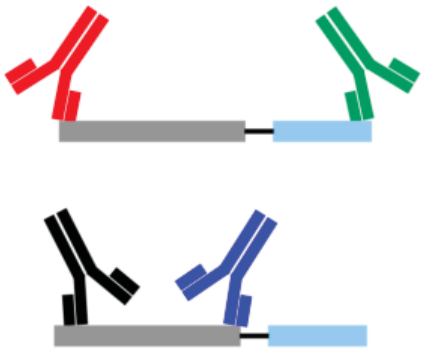
Both cTnT and cTnI are subjected to proteolytic degradation and their proteolytic fragments are present in cardiomyocytes and in plasma. For instance, when cardiomyocytes become apoptotic following HF or an MI, they activate proteolytic enzymes such as caspase-3 and μ-calpain/calpain-I. In addition, phosphorylation of cTn by protein kinase C (PKC) increases whereas protein kinase A (PKA) reduces the proteolytic activity of calpain [44].

The detection of proteolytic fragments by immunoassays is dependent on the design of the catch and detection antibodies. In principal, if one uses a pair of antibodies that bind close to the N-terminal end and close to the C-terminal end of a protein, the resulting immunoassay will only detect full-length proteins. This was most likely the reason behind reports that suggest that only full-length cTnT was present in patient plasma [45-47]. In addition, size exclusion chromatography often fails to resolve full-length cTnT from its

degradation products. The literature on the degradation products formed after an MI and other conditions is therefore complex and sometimes contradictory.

Figure 14. ***Design-dependent Assays.***

Above*: The detection of proteolytic fragments by immune assays is dependent on the design of the catch and detection antibodies. In principal, if one uses a pair of antibodies that bind to the very N-terminal and to the very C-terminal of a protein, the resulting immunoassay will only detect full-length proteins.* ***Below:*** *hs-cTnT and hs-cTnI antibodies from Roche and Abbott binds closely enabling detection of degraded fragments.*



However, if one uses antibody pairs that bind close together, like the Roche hs-cTnT and Abbott hs-cTnI assay used in our studies, most degradation products will be detected. The use of antibody pairs that binds closely to each other on the protein of interest, and their ability to detect most degradation products, are two reasons behind the remarkable sensitivity of the Roche and Abbot clinical cTn assays. We now know that most of the cTnT and cTnI measured by our clinical assays are degradation products in patients with stable cTn elevations a few hours after an MI [23,25,47-49].

In fact, it has been suggested that the development of immunoassays that can detect intact, fragmented, or phosphorylated cTnT separately would be of clinical interest [24], and based on [47] the clinical desire for a C-terminal cTnT detection assay enabling clinicians to distinguish between MI and low-molecular circulating fragments in patients with stable increases of cTnT (called "myocardial injury" by ESC [1]).

The reason why most cTn in the circulation are degradation products has not been fully elucidated. It has long been known that a 29 kDa fragment of

cTnT, lacking over 70 amino acids from the N-terminal is produced in living, but stressed, cardiomyocytes. This region in cTnT is not conserved among the cTnT isoforms and cardiomyocytes that express this 29 kDa cTnT fragment are fully functional but require higher Ca^{2+} concentrations to contract their sarcomeres, what is called lower Ca^{2+} sensitivity [50]. It has been proposed that the cleavage of cTnT to this 29 kDa fragment is one way that the muscle cells survive during ischemic stress, as their sarcomeres consume less ATP when the Ca^{2+} sensitivity is decreased [50-52].

Most data indicate that cTn degradation is local and not produced in the circulation. When cTn was spiked in cTn-negative serum and plasma and incubated at 37 °C for three days, clear degradation of cTnT was observed in serum but not in plasma, strengthening the hypothesis that cTnT is selectively cleaved by proteases within the cardiac tissue and that the proteases might be inhibited by plasma [25].

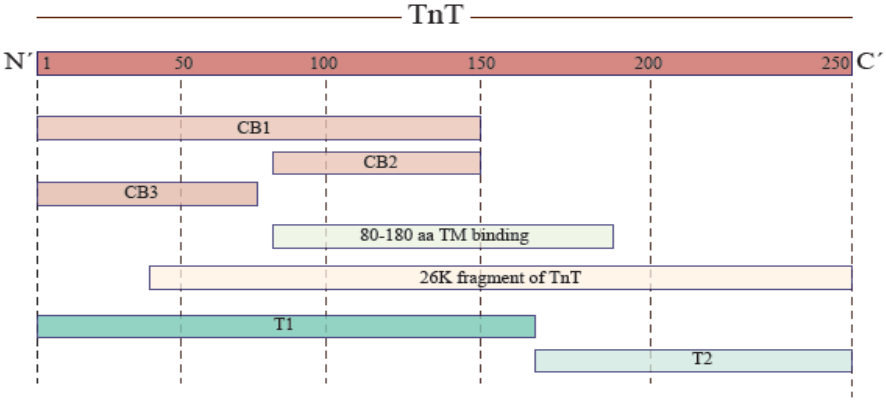


Figure 15. Fragments of Troponin T.

cTnI is also subjected to degradation. Intact cTnI and the primary degradation fragment were found in STEMI patients already 90 minutes after the onset of an MI with further degradation after 165 minutes. This study also observed seven degradation products from both the C-terminal and the N-terminal of cTnI [11,53], in line with other observations [54]. In skeletal muscle, when PKC phosphorylation was performed on skeletal cTn complexes (~90kDa) with a Sephadex G-100, the apparent molecular mass of TnI was reduced from 90kDa to 30kDa, suggesting dissociation from the Tn complex [44]. The primary associated cleaving site from u-calpain is the C terminal that removes residues 194-210 [52].

Necrosis of cardiac tissue, as seen after an MI, is accompanied by the release of different proteolytic enzymes from the lysosomes. For cTnI, it is generally accepted that it is highly susceptible to proteolysis [24]. This degradation is the reason why different cTnI assays with different antibodies and the lack of a golden standard cTnI assay lead to the same plasma sample giving varying results, depending on which antibodies the assays use [24].

1.3.3 Isoforms

Both cTnT and cTnI have several isoforms, meaning that they are expressed from different genes, giving them different properties and unique functions. Below is a summary of what is known about the isomeric forms of cTnT and cTnI.

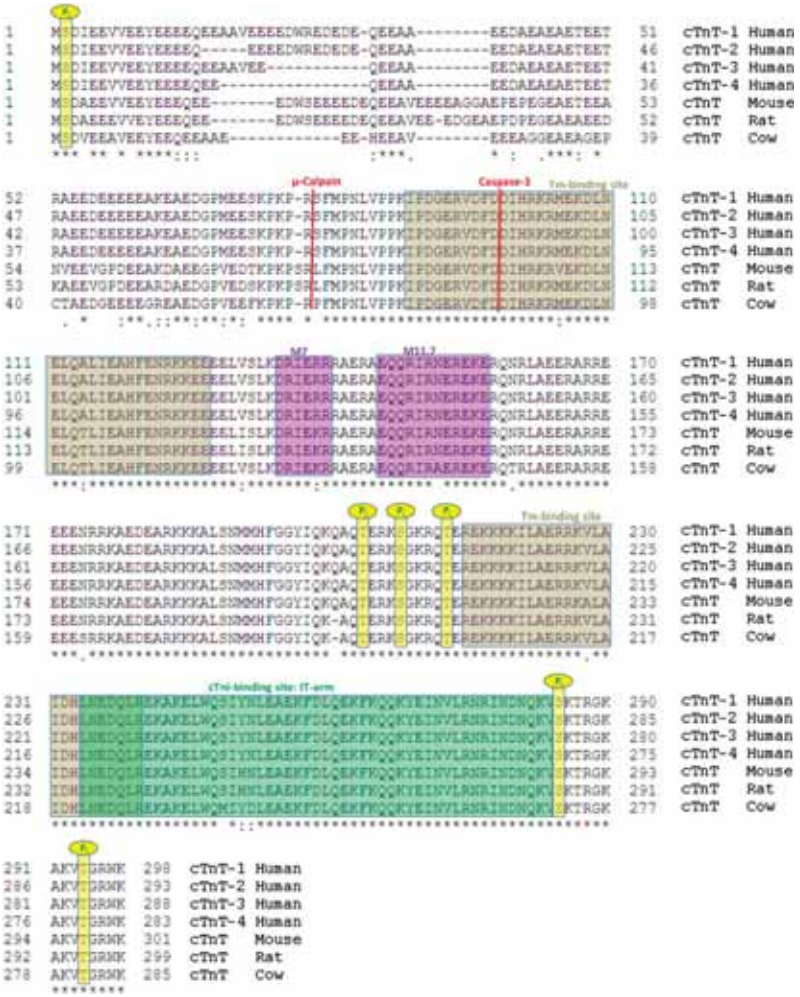


Figure 16. ***Alignment of human, mouse, rat and bovine cTnT isoforms.*** *Human isoforms shown are the fetal isoform (cTnT-1), a second fetal isoform (cTnT-2), the healthy adult cTnT isoform, (cTnT-3) and a third fetal isoform, found to be re-expressed in the adult failing heart (cTnT-4). Mouse, rat and cow cTnT all represent their respective canonical forms. Highlighted sections of the protein indicate various binding sites, phosphorylation sites and proteolytic cleavage sites, as indicated. Purple indicates hs-cTnT assay from Roche.*

Alexander S. Streng,Douwe de Boer,Jolanda et al. Posttranslational modifications of cardiac troponin T: An overview. Journal of Molecular and Cellular Cardiology (2013:63).47-56, copyright © 2020. Reprinted by Permission of Elsevier[24]

TnT: cTnT has 4 isoforms, all expressed from separate genes. The fetal heart has all four isoforms of cTnT with a preponderance of T1 and T4 [51]. During development, T1 decreases and T3 increases, and in the adult heart, T3 is the major form, T4 is the minor form and T1 and T2 are barely detectable. T1 and T2 seem to be the most Ca^{2+}-sensitive cTnT isoform [8]. There is evidence that increased expression of the normally barely detectable T4 might indicate an increased risk of HF or death [52]. Interestingly, there are also some studies that show that the failing heart has increased levels of the T2 isoform [55]. T2 is coded by slow skeletal (ss) TnT, and have decreased maximum ATPase activity in the myofibrils, compared to cTnT, but there is no convincing evidence of re-expression of fetal TnT in failing hearts [52].

TnI: The human heart contains the slow skeletal (ss) TnI isoform in the fetal heart until nine months of age. After this, the cardiac isoform is expressed. The cTnI isoform differs from the fetal form by having an extension on the N-terminal with two serine residues.

1.3.4 Kidney function and clearance

The kidneys regulate the body in several important ways; *e.g.,* by maintaining the acid-base balance, regulating the electrolyte and fluid balance, producing hormones and eliminating waste products when filtering the blood. The filtration occurs in the kidneys' roughly 1 million nephrons. Humans have about 3 liters of plasma and roughly 1 million nephrons. Normal filtration rate is considered to be > 60 ml/minute in a healthy human adult, meaning that the entire plasma volume is completely filtered over 30 times/day. This

is essential in order to maintain homeostasis and patients with very poor renal function need dialysis several days a week to be able to remove low-molecular weight residue products, like urate, creatinine and small proteins like cystatin C, from the cellular metabolism.

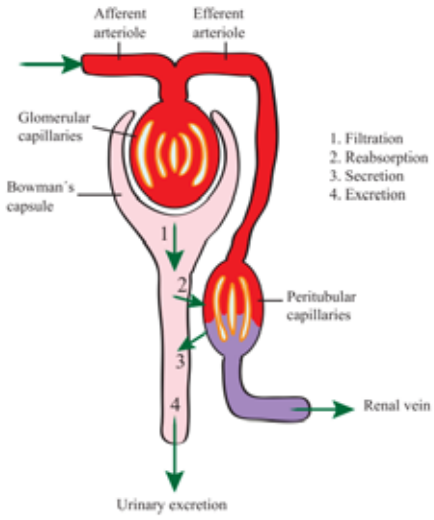


Figure 17. Kidney clearance

The blood enters the kidneys' glomeruli, where 10-20% of all available water and low-molecular components are filtered into the Bowman space. In the proximal tubules; electrolytes, fluid, proteins and other things that the body needs are reabsorbed through active transport back to the bloodstream. In this process, the proximal tubular cells consume large quantities of energy and are, in fact, some of the most energy-consuming cells in our bodies. Molecules that are not taken up by the tubular system are passed to the urine. Over 99% of all water is absorbed so that the more than 100 liters of primary filtrate produced everyday result in 1-2 liters of urine, where unwanted components, like creatinine and toxins, are concentrated.

Glomerular filtration rate (GFR) is a measurement of kidney function and relates to the volume of plasma that is fully filtered in the glomeruli within a certain time. The filtration is driven by tightly regulated higher pressure in the glomerular capillaries compared with the pressure in the Bowman space [56]. By regulating the glomerular pressure, the kidneys can both increase and decrease filtration; that is, a person's GFR may vary significantly between

days as part of this regulation. To enable comparison between different individuals, the GFR is standardized to the body surface of 1.73 m^2, which, at the beginning of the 1900s was the median area of Americans' relative GFR (relGFR) (mL/min/1.73 m^2). To measure the GFR, a cell-impermeable tracer that is neither metabolized nor bound to any structure in the body, is injected i.v. and its clearance is then used to calculate the patient's GFR. Examples of kidney tracers are 51Cr-EDTA, iohexol and FITC-Sinestrin. It is also possible to estimate the GFR based on steady-state levels of creatinine or cystatin C, molecules that are constantly produced and for the most part removed by glomerular filtration. If the kidney function goes down, the steady-state concentration of creatinine and cystatin C goes up. By plotting steady state concentrations from patients where the GFR has been measured by iohexol or 51Cr-EDTA it is possible to find an equation to calculate an estimated GFR (eGFR) by curve-fitting the data. The eGFR is useful, but an imprecise measure of the patient's true GFR, in part, because the calculations assume that all of us produce the same amount of creatinine or cystatin C per unit of weight. This is far from the truth as creatinine is mostly produced by skeletal muscle and different patients have different muscle mass.

1.3.5 Kidney disease and cardiac Troponin

cTn over the 99th percentile, especially cTnT, is common in patients with chronic kidney disease (CKD) [1]. It has long been debated whether the cTn elevation in patients with CKD is due mostly to poor clearance or to increased release from the heart [57]. This debate has been ongoing since cTn was thought not to be cleared by the kidneys. Evidence of that was based on there being no, or very little, cTn in the urine from patients with cTn

elevations [1], and the disappearance of cTn following myocardial infarction being the same in patients with and without kidney function [21,22] in one study. Among patients with CKD, as in all patients, cTn elevation is linked to the development of heart failure and cardiovascular death, the leading cause of early death among CKD patients [40]. As long as the pathophysiology is unknown, we cannot offer evidence-based treatment to these patients [58]. Another problem with not knowing whether cTn elevation among patients with poor kidney function are the false-positive cTn tests in emergency wards, leading to erroneous admissions for having an MI, which results in high costs without actual health improvement.

Interaction between the heart and the kidneys has been known and discussed thoroughly for many decades. The kidneys play an important role in both the initiation and progression of heart failure (HF), and around 30% of patients with HF have renal impairment [57]. Cardio renal syndrome (CRS) is defined by bidirectional crosstalk between the renal system and the cardiovascular system [56]. It usually starts with a primary disorder in one of the systems that leads to dysfunction in the other. For example, dialysis patients with end stage renal disease (ESRD) have a more than tenfold increased risk of cardiovascular death and patients with HF, which leads to acquired renal impairment, are commonly known by cardiologists to have a poor prognosis [56].

HF treatment also leads to impaired renal function [57] and patients with renal disease need to be handled carefully. Increased cTn in HF patients was detected in 1997 and published by two different groups [59,60], and is now accepted as one of the strongest predictors of the future prognosis in patients with HF.

It has been suggested that the cTn cutoffs need to be revised and discussed in relation to age, gender and stage of renal impairment [57]. This has not been done yet, mainly because kidney clearance has never been thoroughly investigated.

1.3.6 Kidney clearance and Troponin

cTnT has a molecular weight of 37 kDa and was therefore not believed to have a high filtration rate over the glomerular membrane. On the other hand, evidence that most circulating cTnT in the body consists of degradation products, 17kDa or smaller [23], indicates that the cTnT found in patient plasma is indeed filtered through the glomerular membrane. Furthermore, cTnT is rod-shaped and not globular, which also supports the possibility that even its intact form could be cleared via the kidneys. In dogs, purified cTn clearance has been assessed as having a 1-2 hour half-life in the circulation, but the involvement of kidney clearance was not fully investigated [14]. Studies of cTnT elimination in patients with myocardial infarction or procedure-induced myocardial damage with different levels of kidney function indicate that kidney function contributes [61], but that extra-renal cTnT clearance mechanisms dominate [62,63].

2 AIM

The goal of my research was to characterize the mechanism of cTn release and clearance after a myocardial infarction.

Specific aims:

Paper 1:

The textbook knowledge asserted that most cTnT was irreversibly bound to insoluble filaments in cardiac tissue and could only be released after degradation of these insoluble filaments in necrotic cardiac tissue.

The aim of the first paper was to investigate cTn release from necrotic heart tissue *in vitro* but using physiological conditions.

Paper 2:

The involvement of kidney function in the clearance of cTnT was not known. The aim of Paper two was to investigate the kidney-dependent clearance of cTnT in patients and rats.

Paper 3:

It has been observed that cTnI reaches higher peak concentrations and returns to normal concentrations faster than cTnT in patients with MI, even though there are similar amounts of cTnT and cTnI in human heart tissue. It had been speculated that the clearance of cTnT and cTnI from the circulation was different. The aims of the third paper were to compare the release and overall clearance of cTnT and cTnI from damaged cardiac tissue and to compare the kidney-dependent clearance of cTnT and cTnI.

3 PATIENTS AND METHODS

3.1 Ethics

The collection of samples and the animal studies in this thesis were performed with permission from the Regional Ethics Committee at the University of Gothenburg, Sweden. Signed informed consent was obtained from each patient when needed and there were no additional risks associated with participation in the study.

3.2 Human biopsies, preparations and samples

In order to study the release kinetics of cTnT from necrotic heart tissue, pieces of human heart, obtained during cardiac surgery, were homogenized in different buffers and plasma and under different conditions to investigate whether physiological conditions could affect the cTnT release. After each extraction experiment, we re-extracted the tissue pellet in large volumes of high-salt buffers that were able to extract all the cTnT from cardiac tissue as a way to estimate the extent of extraction in different buffers and plasma. We also examined the solubility of purified cTnT in different buffers by first drying a cTnT-containing solution at the bottom of a tube and then adding buffers to the dried cTnT and measure the extent of solubilization.

3.2.1 Heart tissue biopsies

Samples of human heart tissue were taken from patients undergoing cardiac surgery at Sahlgrenska University Hospital, Department of cardiothoracic surgery. They were ether biopsies from the auricle of the right atrium (5 x 5 x 4 mm) or left ventricular transmural samples (20 x 20 x 20 mm), healthy donor hearts or the explanted heart from patients with end-stage heart failure

transplantation surgery. The tissues were kept cold and cleared of epicardial fat and cut into small pieces using a scalpel, and frozen at -80°C within 1 hour.

3.2.2 Tissue homogenization

In all the papers, we homogenized heart tissue with a glass douncer that fit snugly to the inner walls of a glass tube, efficiently tearing apart everything that passed the narrow space. This method was chosen after careful examination of the homogenization process of other methods; for instance, the method used by Katus et al. [41]. It was noted that cardiac tissue homogenized in plasma or physiological buffer aggregates into clumps after a brief period of incubation, likely due to reorganization of the sarcomeric filaments. We also found that these clumps retained cTnT and cTnI by what we assume is a "trapping effect". Because tropomyosin-coated filaments reform after homogenization, cTnT that binds tightly to tropomyosin did not escape efficiently from these aggregates. Therefore, the extent of extraction depended on the reformation of these aggregates and we took special care to control this effect during all extraction procedures in this study.

3.2.3 Blood samples from paired kidney veins and arteries

In Paper 2 and 3, patients with moderate renal failure in combination with severe heart failure underwent catheterization of the renal vein and radial artery by fluoroscopic guidance [64].

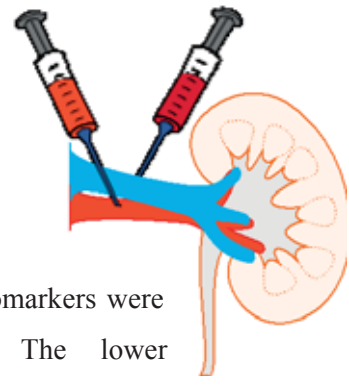


Figure 18. ***Renal catheterization.*** *Measuring the concentration difference in the renal artery and renal vein blood—the kidney extraction index—was calculated.*

The concentrations of cTnT, cTnI and other biomarkers were measured using routine clinical assays. The lower

concentration of cTnT and other biomarkers in the renal vein sample, compared with the sample from an artery, was used to calculate a kidney extraction index. As part of the catheterization procedure, the protocol included calculation of the GFR, plasma renal flow and renal blood flow (RBF). The GFR was normalized to a body surface area of 1.73m^2.

3.2.4 Release kinetics of cTnT and cTnI *in vitro*.

Human, rat and pig heart biopsies were either incubated as 5 mm^3 cubes of left ventricular human heart tissue samples or homogenized in a glass Dounce homogenizer, as described in 3.2, at 37°C or at 0°C for different lengths of time, and the extent of extraction of cTnT, cTnI myoglobin, creatine kinase and aspartate aminotransferase was measured. Following each extraction experiment, we re-extracted the tissue pellet in large volumes of high-salt buffers that could extract all cTnT and other biomarkers from the cardiac tissue, enabling calculation of the total amounts of the tissue biomarkers.

3.3 Animal models and procedures

3.3.1 Animals

All animal experiments in Paper 2 and 3 were performed in male rats since they lack female hormone cycles and male rats are generally calmer and easier to handle. We used both Sprague Dawley rats (Taconic) and Wistar Hannover rats (Taconic) of the same age and weight range. All rats were kept on standard fodder (Envigo) and with free access to water.

3.3.2 Anesthesia

In Paper 2, all animals were anesthetized during the procedures, while in Paper 3 the animals where anesthetized only during injections.

The anesthesia was induced and maintained by inhalation of isoflurane and body temperature was maintained by a heating pad. The reason why isoflurane was chosen instead of pentobarbital was to increase reliability and reproducibility, as isoflurane enables more precise regulation of the anesthetic depth.

The internal jugular veins were catheterized in animals that received bolus injections and continuous infusion of cardiac extracts with a syringe pump and for the collection of blood samples.

Body temperature, blood pressure and hydration levels were all monitored to limit these sources of error. If, for example, the blood pressure is too high, it could have an impact on both clearance and dynamics. At the end of each experiment, the subject animal was humanely euthanized with a lethal dose of isoflurane and the heart was removed.

3.3.3 Kidney clearance rat model

During anesthesia, the abdomen was opened, and the renal arteries were clamped.

3.3.4 Sham treatment

The control animals (called sham) underwent all the surgical procedures except renal ligation. The rationale for this is to remove sources of error of blood pressure and other changes during surgical procedures.

3.3.5 Preparation of rat cardiac extracts

Rat cardiac tissue free from connective tissue and fat was ether homogenized in a 1:2 ratio in rat sera (for steady state and bolus injections), enabling intact forms of cTnT, or incubated overnight (for continuous and discontinuous

experiments), enabling degraded forms of cTnT. The cardiac extracts were supplemented with Co^{2+}-EDTA and FITC-Sinistrin, which are cleared by the kidneys, and human thyroglobulin, a protein with a molecular weight of 600 kDa, served as a marker that is not cleared by the kidneys.

The homogenates were then centrifuged and sterile filtered.

3.3.6 Bolus injection

To simulate a large MI with a defined onset, a large bolus dose of cardiac extracts was injected in animals with and without kidney function.

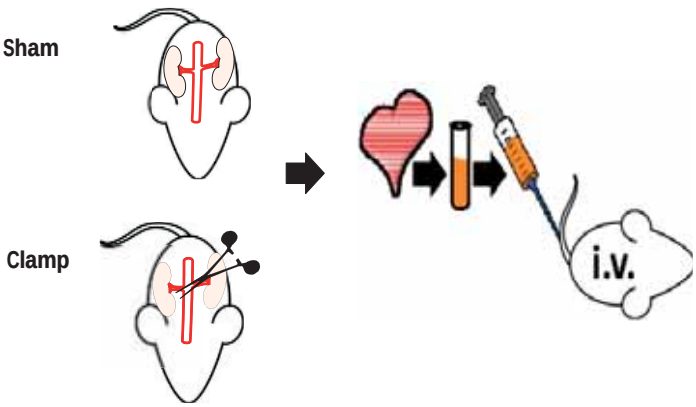


Figure 19. Schematic illustration of Bolus injection.

3.3.7 Continuous and discontinuous infusion

Cardiac extract was given as a bolus injection followed by constant infusion to generate a steady-state situation. When the kidney vessel ligation was performed the infusion either continued or stopped, and blood samples were collected.

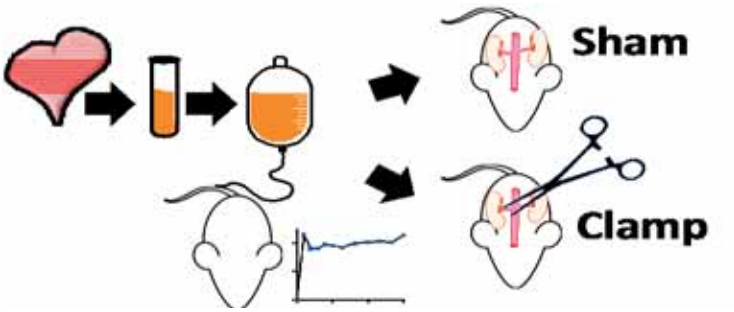


Figure 20. Schematic illustration of continuous and discontinuous infusion

3.3.8 Intramuscular injection of minced rat heart

To simulate myocardial necrosis with a defined onset, minced rat heart supplemented with Co^{2+}-EDTA was injected in the quadriceps muscle in two rats. Blood samples were collected for 168 hours.

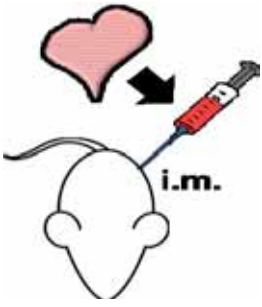


Figure 21. Schematic illustration of Intra muscular injection of minced rat heart

3.3.9 Intravenous and intramuscular injection of rat cardiac extract

To study the clearance of cTnT and cTnI from the circulation, rat cardiac extracts were injected in the quadriceps muscle or the tail vein in rats. Blood samples were collected for 24 hours.

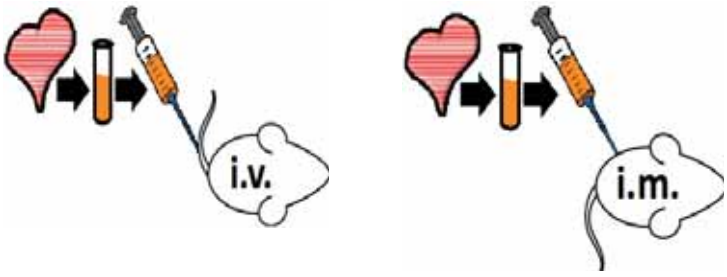


Figure 22. Schematic illustration of intra venous and intra muscular injection of rat cardiac extract

3.4 Laboratory Analyses

3.4.1 Clinical assays

cTnT was measured using the Roche Elecsys® hs-cTnT assay on a fully automated Cobas 602 module at the center for laboratory medicine at Sahlgrenska University Hospital. The within-between and long-term-run CVs have been previously published [32]. When this assay was used, the mean recovery of cTnT from human ventricular cardiac tissue was 0.140mg/g and 0.546 mg/g for rat ventricular tissue (Paper 2, Supplemental table 1). The analyses of creatinine, albumin, sodium, thyroglobulin (Sigma Aldrich) and NTproBNP were performed using the latest version of Roche Cobas®, in which all these analyses have a CV < 5% within the range measured in the studies.

hs-cTnI was measured using the Abbott high-sensitive STAT Troponin-I assay on ARCHITECT, with a CV < 6.9% in this study [65,66].

3.5 In-house manual measurements

3.5.1 ELISA

Myoglobin [1] is an O2-binding protein of 17kDa present in both cardiac and skeletal muscle. Due to its lack of binding partners, it is one of the first measurable biomarkers after a myocardial infarction; however, of short duration.

Rat myoglobin levels cannot be detected by human-specific clinically validated tests and were therefore measured manually using a high-sensitive rat myoglobin ELISA with a CV of 14.9 % at 13.5ug/L (Life Diagnostics). The microtiter well plate contains a monoclonal rat myoglobin antibody. After adding standards and diluted samples to the wells, a horseradish peroxidase (HRP)-conjugated polyclonal myoglobin antibody is added for 1 hour. The wells are then washed to remove the unbound HRP conjugate. Tetramethylbenzidine (TMB), a conjugate for HRP, is added and the samples incubated for 20 minutes, turning positive samples blue. After 20 minutes, a stop solution is added; changing the color to yellow in the wells containing rat myoglobin, and this color is spectrophotometrically measured at 450 nm. The concentration of myoglobin is proportional to the absorbance and is derived from a standard curve.

3.5.2 Flouroscan ASCENT™ FL

FITC-Sinistrin (Frisenius Kabi) is fluorescent-labeled Sinistrin. The fluorescence was measured on a Flouroscan Ascent™ Microplate fluorometer (Thermo scientific) at Excitation 485 and Emission 538.

3.5.3 BIO-RAD protein assay, Bradford method

A simple and accurate method when the sample's soluble protein concentration needs to be determined. It is based on a blue-color change when the Coomassie brilliant blue G-250 dye binds to positively charged amino acids. A standard curve with a known protein content (IGG and BSA) is measured and the protein concentration of interest can then be quantified spectrophotometrically at 595 nm.

3.5.4 Inductively Coupled Plasma (ICP) spectroscopy using Mass Spectroscopy, ICP-MS.

Trace amounts of metals can be identified and quantified through ionization with argon (Ar) gas plasma at high temperature (< 6000K = 5726.85°C). The ICP source inductively heats the gas and converts the atoms of the elements in the sample to ions, which are then separated and detected by MS. The ions enter an electric field and are separated according to their mass/charge ratio. The signal intensity is directly proportional to the concentration of the element in the sample.

3.5.5 SDS-PAGE and analysis of cTnT AND cTnI fragments

(sodium dodecyl sulfate–polyacrylamide gel electrophoresis).

Analysis of degradation products was performed on NuPAGE Bis-Tris gels. The sodium dodecyl sulphate (SDS) detergent binds to the non-polar core in proteins and denatures them, distorting their structure, in the process. To complete this process, dithiotreitol (DTT) is added to break any disulfide bindings that could otherwise retain the proteins structures, and the protein solution is heated to 90°C. After this process of denaturation and SDS saturation, the number of negatively charged SDS molecules per protein is roughly correlated to the molecular weight of the protein, resulting in a uniform charge per weight ratio. After this treatment the proteins become

rod-like and will be negatively charged proportional to their mass and will migrate through the gel towards the positive electrode at a speed proportional to the molecular mass.

After running the gel, gel pieces were cut out and the proteins in them eluted by passive elution. The eluate was then analyzed using clinical assays for cTnT, cTnI and myoglobin, allowing careful quantification of the amount of full-length and degradation products of cTnT and cTnI.

3.5.6 Size exclusion chromatography

Size exclusion chromatography (SEC) is a method that separates molecules based on size. The sample is applied to the top of a column containing porous beads made from Sephacryl. Small particles can enter the pores while large particles cannot and therefore elute faster. Calibration of the 40ml Sephacryl S300 size exclusion column was performed with different molecular weight proteins including myoglobin, human albumin and IgG.

3.5.7 HiTrap CAPTO MMC ImpRes

This ion exchange chromatography (IEX) separates proteins on the basis of their charge and hydrophobicity. The charge in the resin is pH-dependent. Therefore, elution can be performed using a high salt content, high pH or a combination, allowing for specific binding of cTnT and cTnT fragments at pH 7.0, whereas albumin and igG and most plasma proteins are unable to bind under these conditions. cTnT and cTnT fragments are then eluted using 0.1M NaOH, at pH 13. MMC purification typically results in a 50-fold purification of cTnT and cTnT fragments from plasma in one step.

3.6 Statistics

Statistical significance was tested using two-sided unpaired *t* tests and Kruskal-Wallis using Excel 2011; *P* values < 0.05 were considered

significant. Standard deviations were calculated using Excel 2011.

Areas under the ROC curves (AUCs) were compared with the DeLong methodology in MedCalc 14.

4 RESULTS AND CONCLUSION

4.1 Paper 1

The release kinetics of cTnT from necrotic heart tissue were studied in the first paper. Previous studies have shown that cTn is structurally bound to the myofibrils within the cardiomyocyte, with only a 5-10% free fraction of cTnT in the cytoplasm, which would explain the biphasic pattern seen after an MI [13,41]. These experiments were performed *in vitro,* in a buffer that was originally designed to preserve the cTn bound to the myofibrils, and performed at 0°C, which does not correspond to the physiological conditions in the body.

These experiments were repeated mimicking physiological conditions; for example, using plasma and serum at 37 °C and a more efficient homogenization method.

The extraction of cTnT was highly dependent on the ratio of plasma to heart tissue. Using large excesses of serum (434:1) for 30 minutes at 37°C, we recovered around 60% of the cTnT and with three extractions, we recovered 80% cTnT and 99% of the other markers (B). When using equal amounts of serum and tissue, only 3.3% of the cTnT were extracted (C), while other heart damage markers that lack strong binding partners were less affected by the serum ratio.

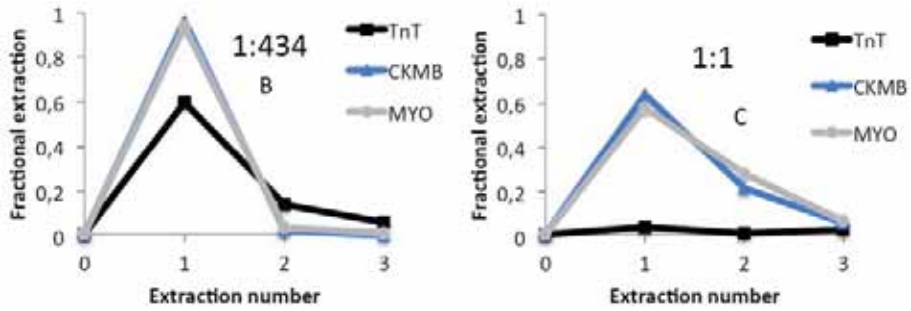


Figure 23. ***Volume dependent extraction of cTnT.*** *(B) Fraction of total cTnT, CKMB, and Myo in human cardiac muscle extracted in 3 repeated serum extractions, each at 37 °C for 30 min at a relationship between serum volume (uL) and human cardiac muscle (mg) of 1:434. The fractional extraction after 3 extractions was 80% for cTnT, 99% for CKMB, and 99% for Myo. (C) Fraction of total cTnT, CKMB, and Myo in human cardiac muscle extracted in 3 repeated serum extraction each at37 °C for 30 min at a relationship between serum volume (uL) and human cardiac muscle (mg) of 1:1. Fractional extraction after 3 extractions was 9% for cTnT, 92% for CKMB, and 93% for Myo*

We also compared the solubility capacity of purified cTn in the previously used cTnT low-salt buffer at 0 °C with human serum at 37 °C (fig 2). The human serum solubilized over 60% of the dried cTnT whereas the low-salt buffer used by Katus et al. [41] was essentially unable to dissolve the dried-down cTnT sufficiently to dissolve cTn.

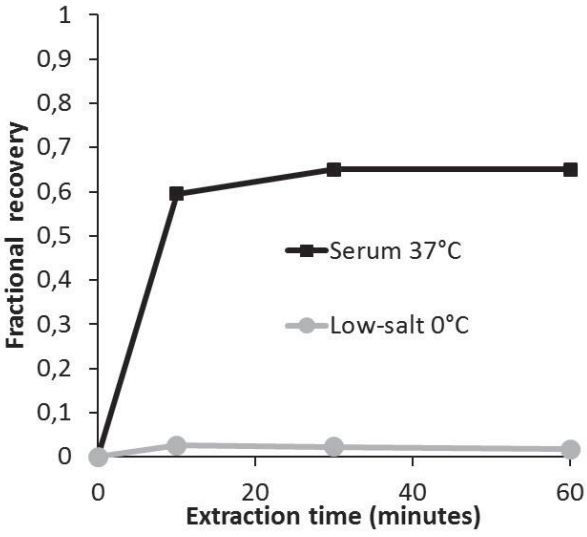


Figure 24. ***cTnT is poorly soluble in the cold low-salt buffer used to define a free early releasable fraction of cTnT.***

Conclusion: The results indicate that cTnT is reversibly bound and that the release of cTnT from a given volume of heart tissue is not static, but highly dependent on the plasma blood flow due to the tissue-trapping effect.

Clinical impact: Our study changes the textbook knowledge concerning how cTnT is released following cardiac necrosis. The knowledge of how cTnT release is affected by the plasma volume allows for a more correct interpretation of changing cTnT levels in patients. For instance, if a patient suddenly displays a second peak of cTnT after a myocardial infarction, our data show that this could, in addition to reinfarction, be due to increased plasma blood flow in the infarcted area, resulting in faster extraction of cTnT.

4.2 Paper 2

Extra-renal clearance dominates at the high cTnT concentrations seen in MI patients.

At increased low levels of cTnT, renal clearance dominates.

There are two major cTn elevation groups: Patients with an acute myocardial infarction (MI), leading to very high levels of cTn, and those without an acute cardiac event, presenting with low but stable, increased cTn levels (< 14ng/L). The latter group more often has poor kidney function, and as many as 35% or more [32,67] of elderly patients in an ED setting have stable elevated levels of cTn without an underlying MI. It is not known if stable elevations of cTn are due mainly to increased release from the heart or to decreased cTn clearance, as the exact mechanisms of cTn clearance were not known.

Therefore, this study focused on possible kidney-dependent clearance of cTnT. To do this, we established a rat model of cTnT clearance using bolus injections of plasma extracts of rat cardiac tissue, to simulate the release of cTnT and myoglobin following an MI. We also examined the renal extraction of cTnT in three heart failure patients undergoing renal vein catheterization. These studies showed that cTnT is efficiently cleared by the kidneys with a clearance rate of 70% of the clearance rate of creatinine. Due to this finding, we examined degradation products of cTnT in plasma from patients with stable cTnT elevations caused by heart failure or decreased kidney function. In accordance with previous studies [23,68], we found that most cTnT measured by our clinical assay consisted of degradation products that are expected to have free passage over the glomerular membrane. Finally, we examined if kidney function-adjusted cTnT levels could improve the diagnostic

performance of our clinical cTnT assay. Using c statistics we found a small but significant improvement of the area under curve (AUC) in an ROC plot when we used kidney function-adjusted cTnT values.

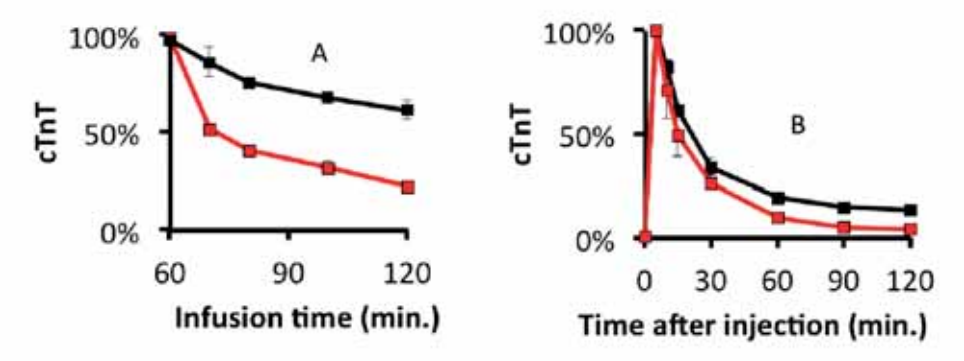


Figure 25. ***Kidney clearance in rats.*** *(A) At slightly increased cTnT levels kidney-dependent clearance dominates. (B) At high levels of cTnT, extra-renal clearance dominates.*

Conclusion: cTnT is cleared in a kidney-dependent manner in both rats and humans. At slightly increased levels, kidney clearance is the dominating clearance process. At high concentrations of cTnT, similar to the levels after an MI, extra-renal clearance dominates.

Clinical impact: We show for the first time that cTnT is cleared by the kidneys. Therefore, part of the cTnT elevations seen in patients with decreased kidney function is due to decreased clearance. Based on our data, it is possible to adjust the cTnT levels to kidney function and therefore make better clinical evaluations of the cTnT levels. For instance, everything else being the same, cTnT is expected to be twofold higher if the kidney function is reduced by half.

4.3 Paper 3

This study focused on the differences in cTnT and cTnI release and clearance mechanisms. Even though these proteins exist in similar amounts in the cardiomyocyte, clinical measurements indicate that there are up to ten times higher levels of cTnI compared with cTnT in some patients with MI, and that cTnI also returns to baseline values faster than cTnT. The reason behind this difference was not known.

Our rat model reproduced the findings in patients with MI. cTnI reached higher levels and returned to baseline levels faster than cTnT after an intramuscular injection of rat heart homogenates. However, when rat cardiac plasma extracts supplemented with cTn were injected intravenously and intramuscularly, cTnT and cTnI behaved identically with regard to release and clearance. The renal extraction of cTnT and cTnI was also cleared simultaneously in seven heart failure patients undergoing renal vein catheterization. The renal clearance of cTnT and cTnI in rats was also the same.

We concluded that the different kinetics and levels in blood samples from patients with MI were likely not due to different clearance of cTnT and cTnI.

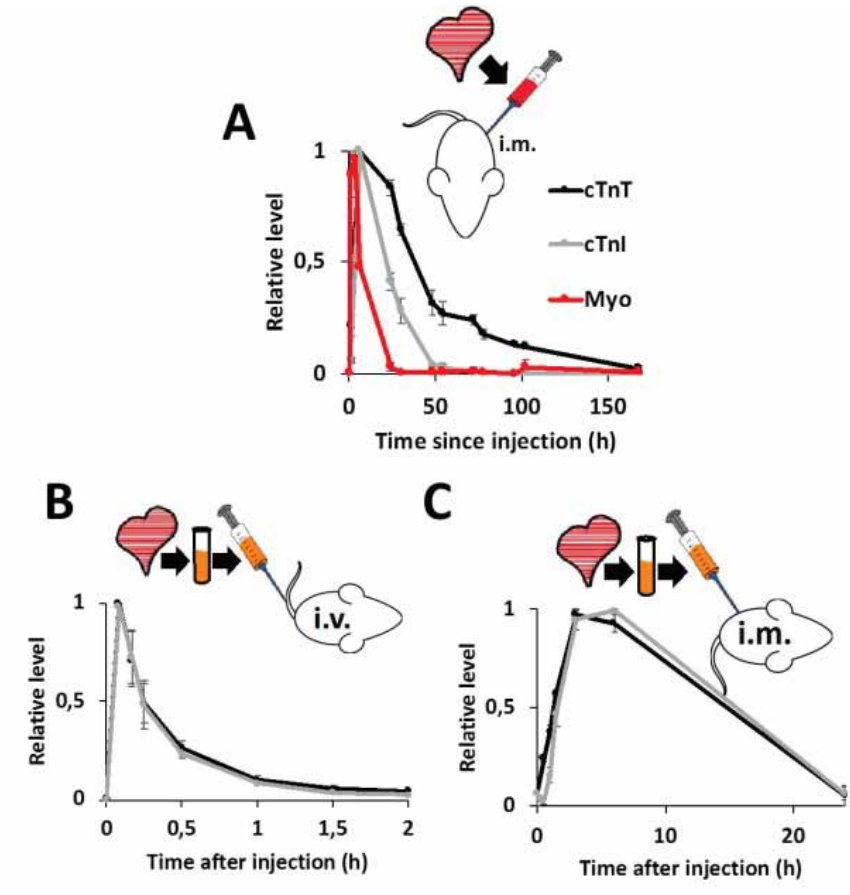


Figure 26. ***Kinetics of cTnT, cTnI, and myoglobin after intramuscular (i.m.) or intravenous (i.v.) injection in rats.*** *(A) i.m. injection of ground cardiac tissue supplemented with the renal filtration marker Co^{2+}-EDTA. Error bars represent the total variation in relative levels between the 2 rats. Kinetics of cTnT and cTnI after injection of cardiac extract i.v. (B) or i.m. (C).*

We therefore turned our attention to the release kinetics of cTnT and cTnI from damaged human cardiac tissue. By using similar experimental setups as in Paper 1, we found that cTnI was released faster and reached higher levels when we simply incubated cube-shaped pieces of human heart tissue in human plasma at 37°C. We found that cTnI reached substantially higher

concentrations than cTnT, and after 20h incubation, only 8% of cTnT was released compared with 82% of cTnI. Using a combination of SDS-PAGE and size exclusion chromatography, we found that the released cTnT and cTnI were mostly degradation products produced by cardiac tissue-resident proteases. We came to this conclusion as high-salt extracts from fresh cardiac tissue almost exclusively contained intact cTnT and cTnI, and when cube-shaped pieces of heart tissue were incubated in plasma, the release was mostly of degradation products and the degradation of cTnI was faster than that of cTnT. Some of the cTnI was released in complex with cTnT and some cTnI was apparently dissociated from cTnT, as cTnT eluted as two peaks in our size exclusion chromatography.

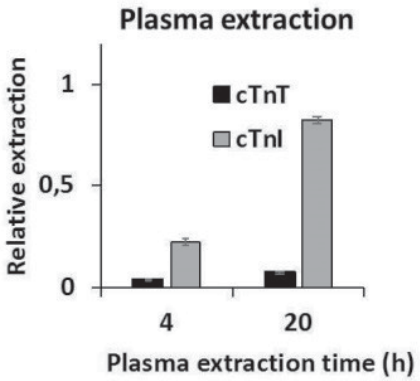


Figure 27. cTnI was released faster and reached higher levels when we incubated cube-shaped pieces of human heart tissue in human plasma at 37°C.

Conclusion: The fact that cTnI reaches higher peak levels and is cleared faster than cTnT in MI patients likely reflects differences in the release kinetics from necrotic cardiac tissue. According to our *in vitro* model, at small relative volumes of plasma in relation to the necrotic volume, the release will be highly dependent on degradation by tissue-resident proteases. As cTnI was degraded faster than cTnT, cTnI is released faster and thereby both reaches higher peak levels and is cleared faster than cTnT.

Clinical impact: In our hospitals, which changed from using a high-sensitive cTnT assay to a high-sensitive cTnI assay in 2019, the clinicians reported that cTnI levels were higher than expected. Our study potentially explains the pathophysiological reason behind this important clinical observation and helps our clinicians interpret the patients' cTnI levels.

5 DISCUSSION

This thesis focuses on how cardiac troponin is released and cleared after a myocardial infarction. The release mechanism and dynamics of troponin from necrotic cardiomyocytes were evaluated in both human and animal studies.

According to the current literature, the first peak of cTnT seen after a large MI would be due to a 5-10% cytosolic free fraction, and the latter, slowly disappearing peak, would be due to degradation of the insoluble myofilaments to which cTn would be irreversibly bound. This was based on the percentage of the cTnT that could be extracted when homogenizing heart tissue in a cold, low-salt buffer originally made to preserve the cTn complex on the myofibrils [21,41,51].

The findings presented here indicate that when large volumes of plasma are combined with a cardiomyocyte homogenate, cTnT and cTnI are released as a complex. When large chunks of cardiac tissue turn necrotic, the plasma-sensitive proteases in the tissue degrade cTnT to smaller fragments, the cTnT-cTnI interaction is broken and cTnI enters the bloodstream as a monomer while the cTnT fragments measured by the Roche assay, which uses antibodies directed to the central core of the major tropomyosin-binding domain, bind and rebind tropomyosin reversibly, resulting in a "trapping effect" not experienced by most cTnI fragments detected by the Abbott cTnI assay (fig 28). This could explain the biphasic peak of cTnT, whereas cTnI only shows a single peak in patients with reperfused STEMI [69]. This could also be the reason why cTnI reaches higher concentrations and return to baseline faster in the same STEMI patients [70].

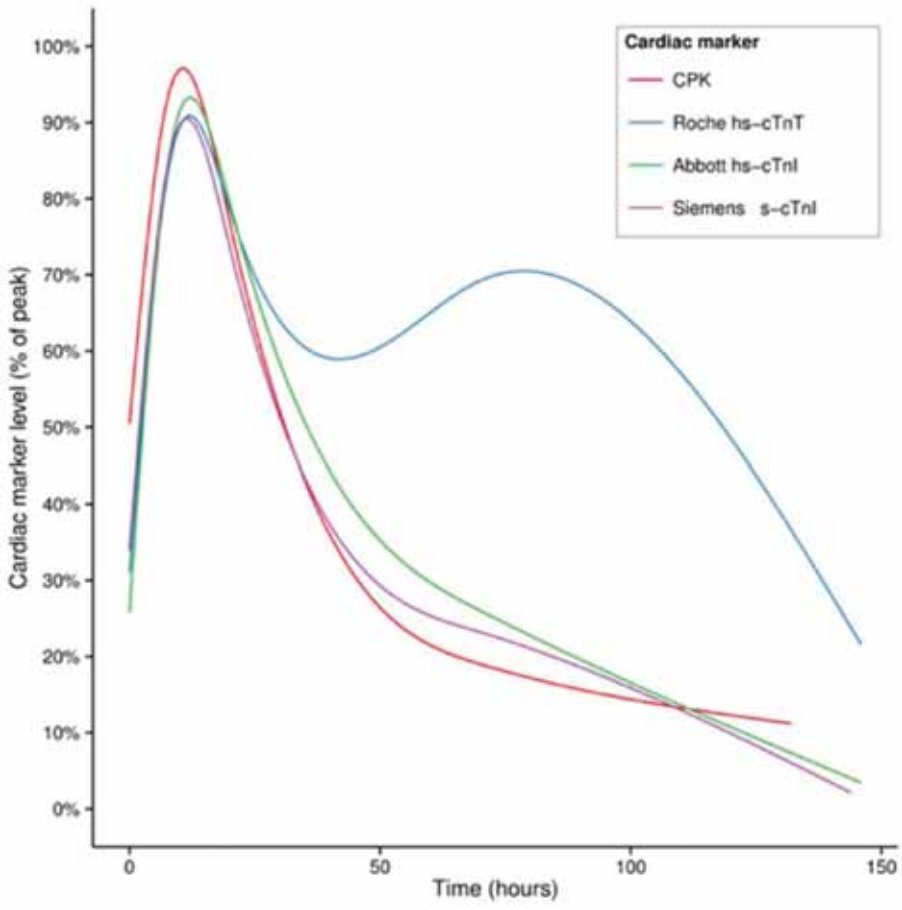


Figure 28. ***Kinetic of cardiac markers since admission predicted by mixed effects models in the whole population, merged.*** *CPK: creatine phosphokinase; hs-cTnT: high-sensitivity cardiac troponin T; hs-cTnI: high-sensitivity cardiac troponin I; s-cTnI: sensitive cardiac troponin I.*

Guillaume Laugaudin, Nils Kuster, Amael Petiton, et al. Kinetics of high-sensitivity cardiac troponin T and I differ in patients with ST-segment elevation myocardial infarction treated by primary coronary intervention. European Heart Journal: Acute Cardiovascular Care (2016:5:4). 354-63, copyright © 2020. Reprinted by Permission of SAGE Publications, Ltd. Reprinted with permission [70]

The cTnT measured by the clinical assay is mostly degradation products with a molecular size expected to allow free passage over the glomerular

membrane. Intact cTnT only exists during the first few hours, at most [23], and after a marathon run, or in patients with ESRD, the most prominent cTnT fragment is around 17kDa in size [49,71]. At low concentrations, kidney-dependent clearance is probably the predominant clearing system. At high cTn levels, such as after an MI, scavenger receptors in the reticuloendothelial system likely dominate the clearance in rats. This is most likely because the scavenger receptors, the "garbage receptors," bind unspecifically to waste in the blood. Likely, the Kd for binding in the scavenger clearance system is in the nanomolar range[72], whereas cTnT levels often are in the picomolar range in patients with stable cTnT elevations, after a marathon or a few days after a medium-sized MI that often peaks around 300 ng/L. In these cases, because the scavenger receptor occupancy is expected to be very low, the scavenger-mediated cTnT clearance is expected to be inefficient.

The efficiency of kidney clearance, on the other hand, which works, in essence, as a diluter of unwanted molecules like creatinine and cTnT, is independent of the cTnT concentration. Therefore, at cTnT concentrations below 100 ng/L, kidney clearance likely dominates. Future studies of the kidney-independent clearance of cTnT will shed light on this question.

The trapping effect

We believe that the slow release of cTnT is due to a trapping effect. After crushing cardiac tissue, insoluble sarcomeres form clumps likely by reassembly of the sarcomere in the solution. It is reasonable to assume that released cTnT in the middle of such cardiac tissue lumps will be able to rebind to thin filaments. Therefore, the cTnT becomes "trapped" by a dissociation-rebinding cycle. This is in contrast to myoglobin that lacks affinity for sarcomeres and is therefore efficiently washed away. This trapping effect is likely a strong factor that allows cTnT to be retained in the

infarcted myocardium, resulting in slow washout after cardiac necrosis. This effect will be large if the necrotic volume is large, and small—even unnoticeable—if the necrotic cardiomyocytes are scattered. This could potentially explain the rapid washout seen after small isoproterenol-induced type II infarctions, a model of the broken heart syndrome in rats, where most necrotic cardiomyocytes are scattered throughout the apical region of the heart and cTnT elevations only last for 24 hours [73].

Proteolytic degradation of cTnT and cTnI limits the trapping effect. Our data suggest that cardiac tissue-resident proteases cleave cTnT and cTnI and that this result in dissociation and release of cTnI faster than for cTnT, since free cTnI has no affinity for thin filaments, cTnI diffuses through the necrotic tissue without being affected by the trapping effect. In contrast, our data indicate that most cTnT fragments measured by the Roche assay seem to retain their ability to bind to necrotic cardiac tissue, likely because the Roche antibodies recognize the major tropomyosin domain. Therefore, although proteolytic fragments of cTnT are produced, they are still affected by the trapping effect. We believe that the faster degradation of cTnI and the fact that proteolytic fragments of cTnT are affected by the trapping effect are the reasons why cTnT is more slowly released.

In Paper 3, this local degradation was investigated in greater detail. In part, the incentive for this study coincided with hospitals in our region switching at this point in time from an hs-cTnT-based diagnostic test to one based on hs-cTnI for suspected MIs. The clinicians reported that, based on their cTnT experience, the cTnI levels were sometimes ten times higher than they expected, even though, according to our data, there are roughly the same amounts of cTnT and cTnI in human cardiac cells and they are cleared similarly, once in the circulation.

Our results revealed that cTnI is rapidly degraded and released faster than cTnT and that the cTnT-cTnI interaction is broken in the process. Initially, cTnT is released as a cTnT-cTnI or cTnT-cTnI-cTnT complex and also released and rebound with high affinity for available tropomyosin in the insoluble thin filaments. cTnT is the only binding partner of TnI. It is this affinity, in a volume-dependent manner, that can be observed as a long plateau phase in patients with non-reperfused MI who usually have as little as 0-5% of normal blood flow [74]. When spiked serum was injected in the tail vein or quadriceps muscle in rats, there was no difference in cTnT and cTnI clearance rates, but when homogenized heart tissue was injected into the hind limb to simulate a large MI, Myo was cleared first, followed by cTnI and lastly cTnT, as expected.

Problems

The results suggest that hs-cTnT and hs-cTnI tests should sometimes be run in parallel. This would, for example, be beneficial when it is necessary to determine whether suddenly increased cTn values are due to reinfarction or increased local blood flow in a previously occluded area, leading to sudden increased washout. High hs-cTnT but low hs-cTnI should be interpreted as no new ischemic event occurring, based on the data that show hs-cTnI to have a higher peak and to be cleared faster than hs-cTnT [75].

Even though elevated cTn values indicate myocardial cell injury, they do not tell us anything about the underlying mechanism. This becomes apparent when studying cTn release in athletes during both long-term [33] and short-term exercise [76]. As little as one hour of bicycle exercise in healthy individuals can double the amount of circulating cTnT, and may even elevate cTnT to

pathological levels [76]. This is an important consideration when patients seek medical attention for chest pain [33]. When Mingels et al. [25] spiked serum and heparin plasma with cTnT, the cTnT in the serum was degraded in a time-dependent manner, but in heparin plasma, the cTnT was not degraded. This indicates that proteases targeting cTnT are still active in serum but not in heparin plasma, and the results thus suggest that the degradation process must occur locally, *in vivo* [25]. These results differ, in part, from those published by other groups. A possible explanation may be that different degradation results may be due to calibration issues, where separation of globular proteins can lead to exaggeration of the protein size, leading to overestimation of the fragment size. When intact cTnT elutes with a larger molecular weight than its actual intact size, the smaller degradation products may appear in a range around which the intact form is supposed to elute, if the assay works properly.

6 FUTURE PERSPECTIVES

This thesis leaves two major unanswered questions:

1. What is the nature of the extra-renal cTnT clearance? We have actually answered this in a study that is accepted for publication. In summary, at high cTn levels found after large AMI 70% of the clearance of cTn occurs in the rat liver and 25% in the kidneys and that liver cells are able to take up cTn by endocytosis. The exact receptors and mechanism involved are under study.

2. What is the nature of the tissue resident protease(s) responsible for the rapid degradation of cTnT and cTnI. This study is ongoing. We will also carefully characterize the tropomyosin-binding properties of the major cTnT

degradation products recognized by the Roche hs-cTnT assay using purified tropomyosin or purified thin filaments, where actin filaments are coated with tropomyosin. This will teach us more about the complex release kinetics of cTnT fragments after local proteolysis.

After that we think that we are almost done with this research project and will move on to explain the pathophysiological mechanisms behind stable cTn elevations, but that will be a very different story.

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